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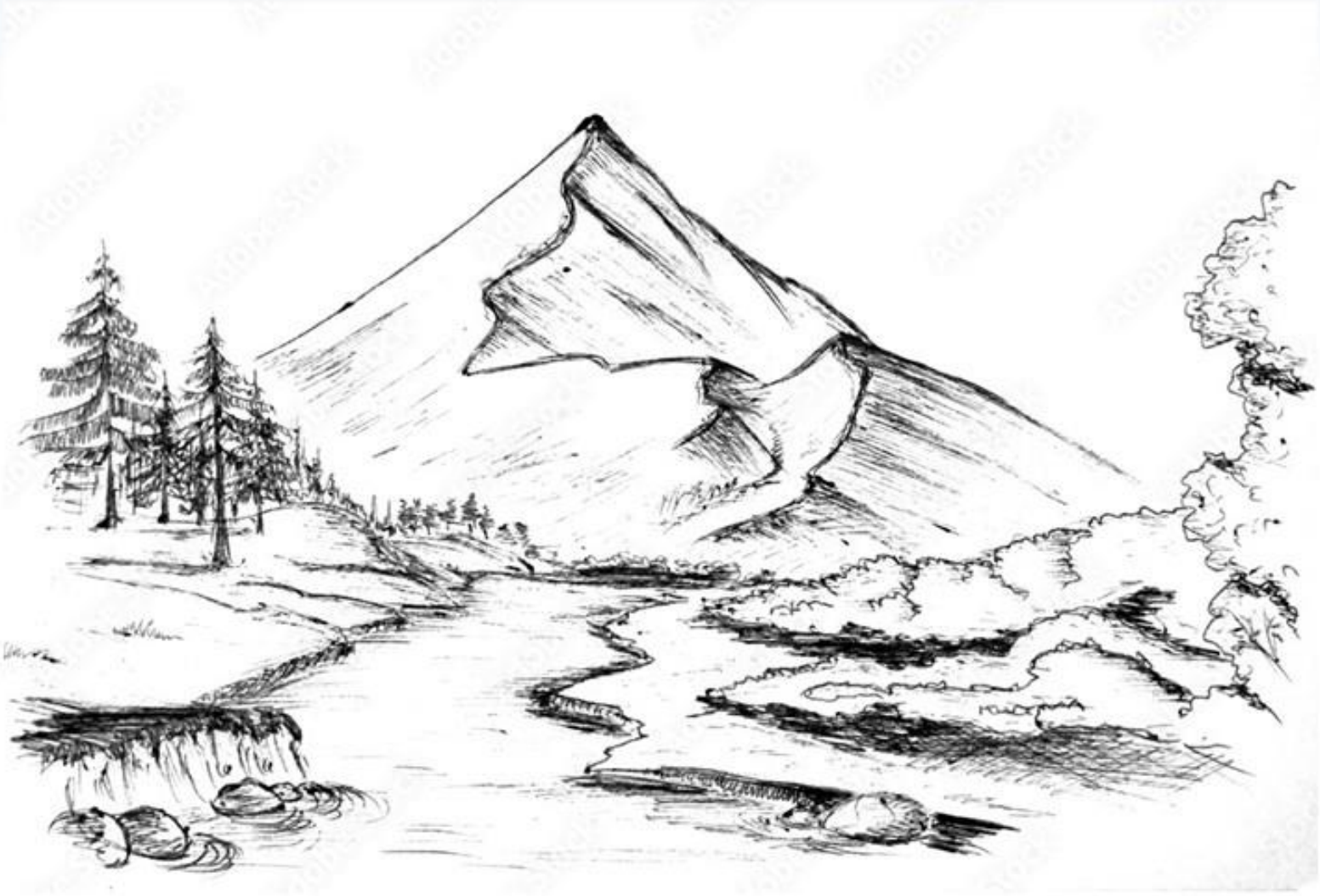

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PART I: RESEARCH BRIEFS

PART II: SELECTED FULL PAPERS

Microbial communities in subsurface flow wetlands

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Abstract

Microbial communities are responsible for the majority of the removal of organic matter in wetlands, making them a key factor for good system performance. However, not many studies performed on wetlands have focused on this aspect. In this paper, a case study is presented, where the microbial communities colonising two laboratory-scale wetlands, one unplanted and one planted, were studied. The functional groups of bacteria present in the two wetlands were found to be very similar, consisting mainly of SRB and methanogens. However, diversity analysis showed that the two systems did not have similar bacterial species, which indicates that systems sharing the same functional bacterial groups cannot be assumed to have equivalent bacterial species.

Keywords*: CW, microbial communities, wastewater, bacteria, SRB, removal*

1. Introduction

Wetlands are used all over the world to remove organic matter, nutrients, metals, suspended solids and pathogens from domestic wastewaters from small or large communities (Nguyễn Hoàng Nam, 2019). They have also been used as part of a treatment chain, for the management of agricultural and farming runoff (e.g. swine and dairy effluents; (Vymazal, 2015)), for the treatment of industrial effluents (e.g. refinery wastewaters, electroplating effluent, textile production; (Maiga, Sperling et al., 2017)), acid mine drainage (Nam Nguyen Hoang, 2011) and high strength effluents (e.g. landfill leachate; (Mulamoottil, McBean et al., 2019)). With all these unquestionable advantages, wetland creation and restoration is being widely promoted across a wide range of climates and geographical locations (Zamora, Marín-Muñíz et al., 2019).

The vast majority of the soluble organic carbon that is removed in wetlands is done so by the microbial consortia, which remove 50 to 95% of the organic matter (both aerobically and anaerobically;(U.S.E.P.A., 2019)), since the uptake by the macrophytes is insignificant (Nguyễn Hoàng Nam, 2019, U.S.E.P.A., 2019, Kochi, Freitas et al., 2020).

In wetland systems, the microbiological transformation of pollutants is aided by energy from the sun and wind, by the presence of plants, animals, by the soil and by the large area of these systems. Hence, constructed wetlands can be used as a low cost, natural technology for

wastewater treatment, requiring very small amounts of non-renewable energies and no chemicals (Nguyễn Hoàng Nam, 2019).

2. Microbial communities in wetlands

Wetlands and other aquatic systems are suitable habitats for the development of large communities of microorganisms (Nguyễn Hoàng Nam, 2019). These microbial communities are responsible for most of the removal of soluble organic matter in wetlands. Therefore, in order to optimise the performance and avoid failure in these systems, it is crucial to have an understanding of the factors determining the structure and function of the microbial communities that carry out the organic matter removal (Rajan, Sudarsan et al., 2019, U.S.E.P.A., 2019, Gajewska, Skrzypiec et al., 2020). However, there is considerable lack of information about the communities of microorganisms inhabiting wetlands and processing the treatment of wastewater, which considerably limits the optimisation of these systems (Badhe, Saha et al., 2014, Nam and Kuschk, 2016, Rajan, Sudarsan et al., 2019).

The microbial communities are mainly composed of bacteria and fungi. Fungi are very important in wetlands, where they are commonly found growing in dead and decaying plant litter. They make a significant proportion of the carbon and nutrients available to plants and algae and, if they are inhibited (by high concentrations of toxic materials), this nutrient cycling is reduced, hence inhibiting the primary productivity of algae and plants. In addition, their symbiotic association with primary producers increases their host's capacity for sorption of nutrients from air, water and soil (Nguyễn Hoàng Nam, 2019). However, the role of fungi might not always be that prominent, as evidenced by preliminary work carried out by Boon *et al.* (1996) on two wetlands in Australia showed that the fungal community was not a significant part of the microbial communities in those systems, despite the large amounts of decaying plant material.

As previously mentioned, bacteria in wetlands are responsible for the majority of the degradation of organic matter in aquatic systems and the flow of carbon, nutrients and energy along the food web, all the way to the higher trophic levels, is ruled by the efficiency of this degradation (Vymazal, 2015, Mora-Orozco, González-Acuña et al., 2018). In wetlands, the bacteria are normally attached to solid surfaces forming a biofilm, which is a heterogeneous microbial community that produces a protective gel that consists of slime-like extracellular polymeric substances (Vymazal 2015, Nam and Kuschk 2016). The microenvironments developed by this matrix protect the biofilm from dramatic changes in the environmental conditions (Rajan, Sudarsan et al., 2019). It can also increase intracellular interactions and the surface area of individual cells, allowing for greater nutrient uptake, and its variable charged regions can attract charged particles like ions and humic acids, increasing the biofilm's capacity

to take up nutrients from the wastewater (Nguyễn Hoàng Nam, 2019, Rajan, Sudarsan et al., 2019). However, the exopolymer matrix can hinder the diffusion of nutrients through the biofilm to the cells, limiting the substrate conversion rates (Rajan, Sudarsan et al., 2019).

Most of the microbial degradation of the pollutants in wastewater takes place close to the solid surfaces (sediments, medium, litter and below ground plant parts) in wetland systems, since that is where most of the biofilm is located (Nguyễn Hoàng Nam, 2019 and U.S.E.P.A., 2019). Biological degradation within the bulk wastewater in wetland systems also occurs, but at very low rates, since the bacterial numbers there are very low (Kadlec, 2019).

Microbial degradation of organic matter is largely affected by the quality of the organic matter and by its residence time in the system. Highly biodegradable substrates are effectively and quickly mineralised, while refractory organic compounds need large residence times to be even only partially decomposed (Nguyễn Hoàng Nam, 2019). Additionally, the organic matter affects the environmental conditions in the system, subsequently having an effect on the microbial activities. In fact, in sediments rich in biodegradable organic matter, dissolved oxygen is only available in the top millimetres near the surface and near plant roots, so that the mineralisation of most of the organic matter is carried out anaerobically (Nguyễn Hoàng Nam, 2019).

In general, the largest molecules (proteins, lipids and carbohydrates) are too large to penetrate the cell membrane of the bacteria and so are broken down to smaller compounds (peptides, soluble saccharides and fatty acids) by hydrolytic enzymes excreted by primary fermentative bacteria. These smaller compounds are then completely degraded to carbon dioxide if alternative electron acceptors are present or broken down to carbon dioxide and low molecular weight compounds (volatile fatty acids and alcohols) by primary fermentative bacteria, when external electron acceptors are limited. Secondary fermenters, namely syntrophic bacteria (which use hydrogen as electron acceptor) or some groups of sulphate reducing bacteria (SRB), then degrade the fatty acids and alcohols to acetate, formate and carbon dioxide. Terminal bacteria such as SRB and methanogens, completely mineralise the products from this last reaction to carbon dioxide and sulphide or methane, respectively (Nam 2011, Nguyễn Hoàng Nam 2019). These processes are shown schematically in figure 1.

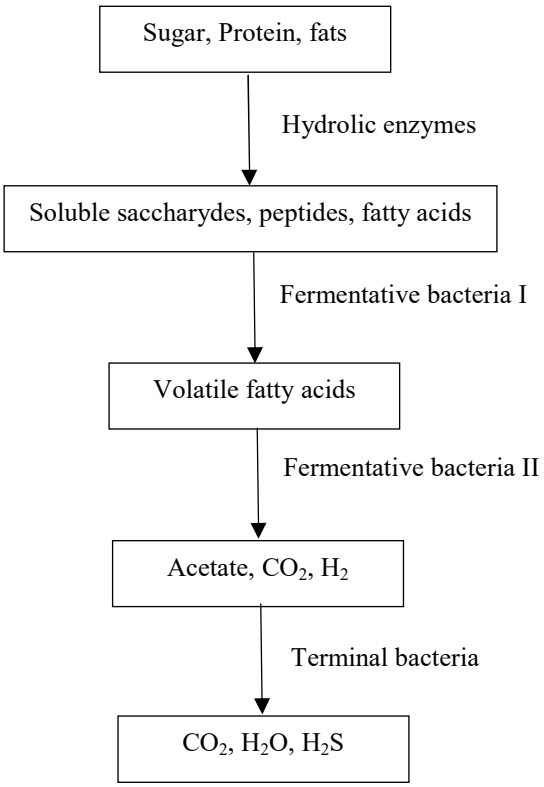

Figure 1. Anaerobic degradation of organic compounds. MOLECULAR TECHNIQUES

Until recently the identification and characterization of microbial communities in the environment was not a straightforward task. The study of microorganisms relied greatly on culture techniques and since the majority of microorganisms cannot be cultured (Head, Saunders et al., 1998), relevant microbial studies were difficult. If we consider that the prokaryotic species have been evolving for more than 3.8 billion years and that they are present almost anywhere in the planet, we come to the conclusion that what we know about microbial diversity through culture techniques is only a minute fraction of that really present (Hugerth and Andersson 2017). In fact, only a very small fraction of the total estimated microbial population has been cultured (Head, Saunders et al., 1998, Hugerth and Andersson 2017, Nguyễn Hoàng Nam, 2019). Estimates of the organisms that have been cultured range widely between 0.1 to 10%, which also indicates how little is yet known about the microbial world (Head, Saunders et al., 1998). Therefore, methods that provided information on the diversity and function of microorganisms were needed, which did not involve the cultivation of microorganisms (Hugerth and Andersson, 2017).

Molecular techniques started being used in the mid eighties and are now widely applied to determine the phylogenetic diversity of microbial communities in the environment (Head,

Saunders et al., 1998, Hugerth and Andersson, 2017). With these techniques, it is possible to compare spatial and temporal changes in microbial communities, which had not been possible in the past with culturing techniques (Pesciaroli, Rodelas et al., 2015). The use of molecular techniques has raised the awareness of how vast the prokaryotic kingdoms really are and how little is still known about them.

Characterization and identification of the microorganisms can be carried out using polymerase chain reaction (PCR) followed by either cloning and sequencing the nucleic acid (and subsequent comparison with a public sequence database e.g. the Ribosomal Database Project, RDP;(Cole, Chai et al., 2003, Pesciaroli, Rodelas et al., 2015)) or 'community fingerprinting' (e.g. denaturing gradient gel electrophoresis - DGGE) and sequencing (Huys, Vanhoutte et al., 2008). Microbial diversity can be estimated by using PCR followed by cloning and sequencing; or by using fingerprinting techniques followed by statistical comparison of the results (Huys, Vanhoutte et al., 2008, Pesciaroli, Rodelas et al., 2015). Microbial identity and abundance can be studied using fluorescence *in situ* hybridisation (FISH), membrane hybridisation, quantitative polymerase chain reaction (qPCR) or, in some instances, most probable number (MPN) technique (Head, Saunders et al. 1998) (Head *et al.*, 1998). Rates (e.g. growth rate) and activity remain the hardest parameters to be studied and estimates can be obtained using culture techniques, chemical techniques and radioactive techniques (Morales and Holben, 2011).

Statistical analysis and ecological theory

The microbial diversity, based on the abundance and positioning of the bands in the DGGE gel, can be analysed using different statistical methods, one of which is based on the theory of random community assembly (Raup and Crick, 1979), used successfully by Curtis *et al.* (2002), Rowan *et al.* (2003) and Isazadeh et al. (2016) (Curtis, Rayne et al., 2002, Rowan, Snape et al., 2003, Isazadeh, Jauffur et al., 2016). Random community assembly suggests that the composition of microbial communities is not fixed in space or time, which means that two reactors treating the same wastewater do not necessarily contain the same microbial communities and even the same reactor cannot be assumed to have the same microbial diversity at two different points in time (Rowan, Snape et al. 2003, Nam, 2011).

Raup and Crick developed a method to predict how many species can be expected to be shared between two data sets and the expected variation in this number. They used a sample randomization procedure (Monte Carlo simulations), where (at least) two community data sets were compared against a random set of data, resulting in an index of similarity between the different samples. This index of similarity is equal to the probability that the expected similarity between different samples is equal to or less than the observed similarity. Based on the value of the index of similarity, if two communities are more similar than predicted by the null

hypothesis, it means that there was a positive bias in the assembling of the communities and, conversely, if the samples are more different than predicted, it indicates that there was a negative bias in the make-up of the communities (Raup and Crick, 1979).

Case study

Two laboratory-scale wetlands, one planted with *Phragmites australis* and the other one unplanted, were operated for a period of one and a half years being fed with diluted beer with an average COD concentration of 385 mg/l and an average sulphate concentration of 76 mg/l. The initial COD/sulphate ratio was quite low and favourable for SRB, but after the first 3 months of operation, it increased to values well above the optimum for SRB. Most of the organic matter was present as acetate and propionate.

Based on FISH and DGGE analyses, bacteria were found to be the entities responsible for the removal of carbon in the two wetlands. In fact, incomplete oxidisers (SRB) degraded most of the propionate in the wetlands and methanogenic bacteria degraded most of the acetate, both being responsible for at least 90% of the TOC removal and around 70% of the COD removal in both wetlands. The microbial communities around the plant roots consisted mainly of methanotrophic bacteria and SRB (both complete and incomplete oxidizers). These results suggest that both methanogenesis and sulphate reduction coexisted quite well in the sediments from the wetlands. However, if the COD/sulphate ratio had been low in the feed, the majority of the acetate would have most likely been degraded by acetate-degrading SRB, since these have a higher affinity for this substrate than methanogenic bacteria (Winfrey and Zeikus, 1977). If SRB had degraded most of the acetate, the performance of both systems would, most likely, have been better, since SRB have higher growth rates than methanogens, consequently degrading larger quantities of substrate per unit time. However, increased amounts of hydrogen sulphide would be produced and would need careful monitoring, as previously found by Nam, Nguyen Hoang and Nam an Chung (Nam, 2011, Nam and Chung, 2015).

One of the rare studies carried out on the composition of the microbial communities in wetlands was developed by Boon and Sorrell in 1991, when they detected the presence of methanogens, methanotrophs and SRB in a permanently flooded wetland in Australia. These researchers also found that methanogenesis and sulphate reduction coexisted in the wetland (Boon and Sorrell 1991). Then, in 1996, Boon and co-workers used that same wetland and found that methanogens formed as much as a third of the total community of prokaryotes. Methanotrophs and SRB were also detected and their abundances were estimated to be 2% and 0.5%, respectively, of the total prokaryote population. Similarly to the study here described, methanogenesis was found to be one of the main processes of organic degradation, being responsible for over 60% of the carbon mineralization (Boon, Virtue et al. 1996).

The analyses of the diversity of the microbial populations in the two lab-scale wetlands produced some unanticipated results, since plants were expected to influence the make-up of the microbial communities. However, the bacterial communities in the two wetlands were assembled randomly, suggesting that the microbial communities in the two systems were no more similar or different than they would be had they been assembled solely by chance. Additionally, the analyses showed that comparison of the diversity of the eubacterial communities in the wetland samples with the feed, mostly yielded similarity indices between the upper and lower confidence limits of the null hypothesis. This evidence suggests that the introduction of a variation in the environment (plants/no plants) did not result in deterministic changes in the microbial communities, since there were no consistent spatial or temporal differences or similarities in the microbial communities. On the other hand, even though both systems had similar numbers of SRB and methanogens amongst themselves, that did not result in both wetlands sharing similar species of these two major functional groups.

These results corroborate the idea developed by Curtis *et al.* that identical ecosystems cannot be assumed to share the same microbial communities, even when performing in similar ways (Curtis, Rayne et al., 2002). Also, the similar behaviour of the two systems in terms of pollutant removal could be due to each of them containing physiologically different but functionally similar groups of microorganisms, or the two systems could have had different microbial diversities, but could both have common microorganisms which were functionally dominant in the two systems. In this study, the first hypothesis seems to be more viable since there did not seem to be a consistently dominant organism in the samples analysed.

Hence, the conditions in the wetlands determine the functional groups of organisms that will be present, but the species within those groups invade the reactors according to stochastic invasion processes. For instance, at the start of this study, the wastewater had low COD/sulphate ratio and the conditions in the wetlands were anaerobic, both of which favoured the presence of SRB. Additionally, the fermentative bacteria present in the wetlands were breaking down most of the organic carbon into propionate and acetate, hence supporting the presence of organisms that use these substrates efficiently. However, the bacterial species within the groups of SRB that consume acetate and propionate and that were present in the feed colonized the wetlands randomly.

Traditionally, wastewater engineering has been based on the assumption that microbial assemblies are determined by external factors, suggesting that similar environments will have similar microbial communities, and different functions in the system are attributed to distinct restricted bacterial species (e.g. (Metcalf and Eddy Inc., 1991)). The results found here hint otherwise, which suggests that modelling and understanding microbial diversities is a key feature

in understanding biological wastewater treatment systems. On the other hand, they are likely to be much more versatile and resistant to changing operating conditions than widely accepted since the microbial communities in the reactors are likely to include a wide range of different microorganisms, probably not all of which are affected by the dramatic changes in the reactor conditions. In reality, the more diverse the microbial communities in a reactor, the more likely they are to resist to perturbed operating conditions (Nguyễn Hoàng Nam, 2019, Gajewska, Skrzypiec et al., 2020). Additionally, two reactors that operate similarly under normal operating conditions might operate completely differently under perturbed conditions, since "minor populations" which are probably distinct in the two systems, are likely to take over when the systems are under stress and result in completely different performances from each reactor(Gajewska, Skrzypiec et al., 2020).

Conclusions

Microbial communities in wetlands are the main entities responsible for the degradation of organic matter. The functional microbial groups present in a given wetland are largely determined by the nature of the feed and by the environmental conditions in that system. However, the species forming each functional group are thought to be no more or less similar than if they had been randomly selected from the source (e.g. feed). Hence, the functional microbial groups present in a wetland can be inferred from the conditions in that system, but the species forming those functional groups can not as yet be known or inferred without carrying out molecular analysis.

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